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(57) Abstract

Tumor metastasis is inhibited by the use of active agents which block infiltration of tumor cells into secondary target organs. These agents include a number of antibodies and various fragments and derivatives thereof as well as hyaluronidase, hyaluronic acid and analogs thereof.

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PREVENTION OF TUMOR METASTASIS

FIELD OF THE INVENTION

The present invention is in the field of tumor metastasis and concerns compositions for preventing invasion of metastatic cells into specific organs.

BACKGROUND OF THE INVENTION

In the course of tumor progression, some of the primary tumor cells going through a series of required changes acquire the ability to detach from the primary tumor mass, penetrate the extracellular matrix and migrate into the lymphatic or blood systems. Some of these cells are then able to penetrate into secondary tissue to form tumor metastasis. The dissemination of malignant cells from the primary tumor also often results from the eradication of primary tumors by surgical intervention or various other treatments such as irradiation. Invasion of essential organs by disseminated tumor cells is the major cause of death from malignant diseases and yet still very little is known about the mechanism of this process and effective treatment for metastasis prevention or blockage is extremely limited.

Results of several experimental systems suggest that adhesion molecules employed by normal cells for traffic and localization in various organs and inflammation sites might be involved also in the dissemination

of malignant cells from the primary site to other tissue[1-3]. The implication of an adhesion molecule belonging to the Integrin adhesion molecule family termed LFA-1, in the invasion of a mouse lymphoma cell line into hepatocyte cultures as well as in their metastatic potential has been shown [4,5].

Another integral membrane glycoprotein CD44, known to be a homing receptor of lymphocytes, was found to be involved in the metastatic potential of various tumor cell lines[6]. Several variants of CD44 (vCD44) having additional extracellular domains have been detected in a variety of tissues and tumor cells. One CD44 splice variant, originally discovered on metastatic cells of a rat pancreatic adenocarcinoma (BSp73ASML), has been shown by transfection assays to confer metastatic behavior to non metastatic tumor cells whereas the standard type CD44 (sCD44) did not[7,8]. A monoclonal antibody which recognizes the extracellular region of the rat vCD44 was shown to inhibit the lymph node and lung colonization capacity of the metastatic adenocarcinoma BSp73ASML.

Several other antibodies which react with various metastasis - specific variants of CD44 were shown to be immunosuppressive in rats[9].

CD44 has been proposed to be the principle cell surface receptor for hyaluronic acid (HA) and CD44 molecular species were found to be bound to HA (although sometimes only after activation with phorbol ester)[10,11].

The nucleotide and amino acid sequences equivalent to the extracellular variant domains in mouse rat and human CD44 have been found and described[12, 13].

LIST OF PRIOR ART

The following is a list of prior art and references considered to be pertinent for the description below.

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The citation herein of the above publications is given to allow an appreciation of the prior art. This citation should not, however, be construed as an indication that this art is in any way relevant to the patentability of the invention, as defined in the appended claims.

The above publications will be acknowledged herein by indicating their number from the above list.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide agents which will inhibit invasion and growth of tumors in organs other than those from which the tumor originated.

The following are meanings of some of the terms to be used in the description below:

"Primary tumor" – a tumor originating in an organ or tissue as a result of a cancerous transformation process of cells in this organ or tissue;

"Secondary tumor" – a tumor which arose as a result of dissemination of malignant cells from the primary tumor, their migration and infiltration into another organ, the term generally also known as *"metastasis"*;

"Primary organ" and *"Secondary organ"* – the organ in which the primary tumor developed and the organ in which the secondary tumor developed, respectively;

"Invasion" – the combined process of migration of malignant cells from a primary tumor and their infiltration into a secondary organ to develop the secondary tumor; and

"Anti-invasion agent" – an agent capable of inhibiting the invasion process.

In accordance with the present invention several novel anti-invasion agents are provided.

It was found in accordance with the invention that an anti-CD18 antibody, is capable of blocking invasion of tumor cells into the spleen.

It was also found in accordance with the invention that anti-CD44 antibodies, particularly such directed against the 80-90 kilo Dalton (kDa) isoform of CD44 (*"standard CD44 - sCD44"*) blocked the invasion of tumor cells into the lymph node.

It was furthermore found in accordance with the invention that anti-VLA-4 mAb in combination with an anti-VACM-1 mAb, blocked invasion of tumor cells into the liver, while each one of them by themselves showed very little efficacy in such blocking, if at all.

Another finding of the present invention concerns blocking of invasion of CD44 expressing cells into lymph nodes by prior treatment with hyaluronidase.

The findings in accordance with the present invention open the way for new therapies for the treatment of cancer in general and for the treatment of cancer patients to avoid metastasis in particular. The above antibodies with the anti-invasion activity can be used as is for blocking tumor invasion into secondary organs. In addition to the use of such antibodies also fragments thereof such as, for example, F(ab) fragments, $F(ab')_2$ fragments, single chain antibodies, as well as various proteins or peptides derived therefrom which have the same binding activity to an antigen as these antibodies may be used for the anti-invasion therapy of the invention. Such a derivative may for example be a molecule derived from an antibody or fragment which comprise an antigen binding site having the same binding specificity as said antibody or fragment and in which one or more amino acid has been replaced, added or deleted.

In addition, the finding of the invention that hyaluronidase has the ability to block invasion of CD44 expressing cells into lymph nodes, may open the way of using hyaluronidase or enzymes having a similar activity in cancer therapy for blocking the occurrence of metastasis in lymph nodes. In addition to the enzymes, also fragments thereof which retain the ability to break down hyaluronidase acid, other agents which have the same activity as well as hyaluronic acids and analogs of hyaluronic acids which are capable of binding to the sCD44 antigen may be used in accordance with this embodiment of the invention.

By one embodiment, the present invention thus provides an anti–invasion agent for blocking invasion of tumor cells into the spleen, comprising an effective amount of an active agent being one or more of the group consisting of:

(a) anti–CD18 antibody;

(b) a fragment of the antibody of (a) comprising an antigen binding site thereof; and

(c) a modified molecule derived from (a) and (b) comprising an antigen binding site having the same binding specificity as said antibody or fragment.

In accordance with a second embodiment of the present invention there is provided an anti–invasion agent, for blocking invasion of tumor cells into lymph nodes, comprising an effective amount of an active agent selected from the group consisting of:

(a) anti–sCD44 antibody;

(b) a fragment of the antibody of (a) comprising an antigen binding site thereof; and

(c) a modified molecule derived from (a) and (b) comprising an antigen binding site having the same binding specificity as said antibody or fragment.

By a third embodiment of the present invention there is provided an anti-invasion agent for blocking invasion of tumor cells into lymph nodes, comprising an effective amount of an active agent being one or more of the group consisting of:

(a) hyaluronidase;

(b) hyaluronidase fragment capable of breaking down hyaluronic acid;

(c) an agent other than hyaluronidase or said fragment capable of breaking down hyaluronic acid;

(d) hyaluronic acid; and

(e) analogs of hyaluronic acid capable of binding to the sCD44 antigen;

By a fourth of its embodiments, the present invention provides an anti-invasion agent for blocking invasion of tumor cells into the liver, comprising a synergistic combination of a first and a second agent; said first agent being one or more of the group consisting of:

(a) anti-VLA-4 antibody;

(b) a fragment of the antibody of (a) comprising an antigen binding site thereof; and

(c) a modified molecule derived from (a) and (b) comprising an antigen binding site having the same binding specificity as said antibody or fragment;

said second agent being one or more of the group consisting of:

(d) an anti-VACM-1 antibody;

(e) a fragment of the antibody of (a) comprising an antigen binding site thereof; and

(f) a modified molecule derived from (a) and (b) comprising an antigen binding site having the same binding specificity as said antibody or fragment.

The amount of active agent to be used in each of the above embodiments should be such so as to achieve the desired clinical effect.

The artisan will have no difficulties in determining the effective amount in each case.

Said active agent can be used in the therapy of various types of cancers so as to avoid the development of metastasis in secondary organs. Various tumors have a tendency of developing a secondary tumor in a well defined organ or tissue and in such cases it may at times be advisable to administer the active agent directly into the suspected organ or tissue or in proximity thereof. The active agent of the invention may be administered to an individual in need during treatment of his primary tumor, e.g. in combination with the administration of chemo-therapeutic agents intended to kill the primary tumor cells. The administration of the active agent of the invention will be typically performed periodically, at least throughout the period of treatment of the primary tumor.

The present invention further provides use of any of the above agents in the preparation of pharmaceutical compositions for use in anti-cancer therapy.

The present invention still further provides a method of cancer treatment for the purpose of avoiding invasion of tumor cells from a primary organ to a secondary organ, comprising use of any of the above anti-invasion agents.

The invention will now be described with reference to some specific embodiments described in the following examples and the appended drawings.

DESCRIPTION OF THE DRAWINGS

Fig. 1 shows FACS analysis of LB T-cell lymphoma cells. The type of stain cells and the specificity of the first layer mAb used for staining are indicated in each panel. The left histogram in each panel shows the non specific binding of the indicator secondary FITC-labeled antibody to various

cell types. The right histogram in each panel shows the specific binding of the first monoclonal antibody to the same cells.

Fig. 2 shows an electrophoretogram of [^{35}S] methionine-labeled LB lymphoma cells which were immunoprecipitated either with a control rat IgG mAb (lane 1 in the figure) or with an anti-CD18 mAb (lane 2 in the figure) and the immunoprecipitate was subjected to SDS-PAGE under reducing conditions.

Fig. 3 shows histology of spleens (a-c in the figure) and lymph nodes (d-f in the figure) of BALB/c mice from the following groups:

(i) non treated mice (a and d);

(ii) mice injected s.c. with $3x10^6$ LB cells (b and e);

(iii) mice inoculated s.c. with $3x10^6$ LB cells and injected i.v. with anti-CD18 mAb (c and f);

all magnifications, x18; and insets, x292.

Fig. 4 shows the incorporation of [^{3}H]-Thymidine in cell suspensions derived from spleens and lymph nodes of BALB/c mice receiving the various treatments indicated in the figure. The results are expressed as cpm (mean ± SD) and each experiment is representative of at least two experiments.

Fig. 5 - Fig. 5A shows the FACS analysis of non treated (A1 in the figure) or PMA treated (A2 in the figure) LB lymphoma cells stained with a first anti-CD44 antibody and a secondary FITC-conjugated antibody. Fig. 5B shows a FACS analysis of direct double staining of LB cells with both an FITC-conjugated anti-CD18 mAb and phycoerythrin-conjugated anti-CD44 mAb.

Fig. 6 shows an electrophoretogram of an LB cell extract which was immunoprecipitated using either the rat IgG antibody (lane 1 in the figure) or the anti-CD44 antibody (lane 2 in the figure) wherein the immunoprecipitates were subjected to SDS-PAGE under reducing conditions.

Fig. 7 shows the histology of the spleen and lymph node of BALB/c mice of the following groups:

(i) lymph nodes (A in the figure) and spleen (E in the figure) of non treated BALB/c mice;

(ii) lymph node (B) and spleen (F) of BALB/c mice inoculated s.c. with LB lymphoma cells only;

(iii) lymph node (C) and spleen (G) of BALB/c mice inoculated with LB cells and injected i.v. with anti-CD18 mAb; and

(iv) lymph node (D) and spleen (H) of BALB/c mice inoculated s.c. with LB cells and injected s.c. with anti-CD44 mAb.

Fig. 8 shows the incorporation of ^{3}H-Thymidine expressed as mean cpm ± SD of cell suspensions prepared from lymph nodes and spleens of BALB/c mice receiving various treatments (as indicated in the graph).

Fig. 9 is a photograph of a cell suspension of spleen cells (A in the figure) and lymph node cells (B in the figure) which were incubated with LB cells at a ratio of 8:1, respectively. The photograph was taken under an inverted microscope wherein the original magnification was x400. Aggregates are seen in the spleen cell suspension (A). The arrows in Fig. A show the LB cells participating in the aggregate (which are discriminated from the spleen cells participating in the same aggregates by their size).

Fig. 10 shows the histology of the liver of BALB/c mice of the following groups:

(i) BALB/c mice inoculated s.c. with H-10 lymphosarcoma cells (A in the figure). H-10 cells are seen infiltrated into the liver parenchima via the blood vessels (the dark area in all the pictures);

(ii) BALB/c mice inoculated s.c. with H-10 lymphosarcoma cells and i.v. injected with anti-VACM-1 mAb (B in the figure);

(iii) BALB/c mice inoculated s.c. with H-10 lymphosarcoma cells and i.v. injected with anti-VLA-4 mAb (C in the figure); and

(iv) BALB/c mice inoculated s.c. with H-10 lymphosarcoma cells and i.v. injected with a combination of anti-VACM-1 and anti-VLA-4 mAbs (D in the figure).

All the magnifications in the figure, x73.

Fig. 11 shows the [^{3}H]-Thymidine incorporation expressed as mean cpm ± SD of cell-suspensions from livers taken from BALB/c mice receiving various treatments as indicated in the figure.

Fig. 12 shows results of an *in vitro* binding assay in which PMA activated LB cells were incubated on a plastic plate containing various substances as indicated in the figure. Results are expressed as percent of adhesion = bound cpm per well/total cpm added to each well x 100.

Fig. 13 shows the Thymidine incorporation expressed in mean cpm ± SD of lymph nodes and spleen cell suspensions from BALB/c mice which were either non treated, inoculated with LB lymphoma cells only or inoculated with LB lymphoma cells and injected with various the enzymes indicated in the figure.

Fig. 14 shows the histology of spleens and lymph nodes of BALB/c mice from the following groups:

(i) mice inoculated s.c. with LB cells only (A in the figure); and

(ii) mice inoculated s.c. with LB cells and injected s.c. with hyaluronidase near the lymph node (B in the figure).

EXAMPLE 1

The expression of the LFA-1 adhesion molecule on T-cell lymphoma LB cells was measured by FACS analysis. LB cells were removed from the ascitic fluid of 6 to 8 week old female mice. Lymph node cell suspensions were prepared by pressing the lymph nodes of BALB/c mice through a stainless steel mesh.

The mAbs (0.1 ml) were added at the appropriate dilution to $3x10^6$ prewashed and sedimented cells. The cells were kept for 30 min. on ice and then washed in RPMI 1640 containing 0.1% BSA. Then, 0.1 ml of species-specific anti-IgG (H and L chain) antibodies conjugated to FITC, diluted 1/20, was added to the sedimented cells. The cells were again kept for 30 mins. on ice, washed in RPMI 1640 containing 0.1% BSA, and resuspended in 0.5 ml of the same medium. The cell suspensions were analyzed in a cell sorter (FACS 440; Becton-Dickenson, Mountain View, CA) tuned to 488 nm. Each cell suspension sample was also analyzed for non-specific binding of FITC-antibody. Specific and non-specific binding of fluorescent antibody was measured at wavelengths above 520 nm. Cell debris was gated out. As seen in Fig. 1, the LB cells express both the α-chain (detected by using the CD11a antibody) and β-chain (detected by using the CD18 antibody) of the LFA-1 molecule.

EXAMPLE 2

The expression of LFA-1 molecules on LB cells was confirmed by the [^{32}S]-methionine immunoprecipitation assay. LB lymphoma cells (10^7/ml) were metabolically labeled with 0.5 mCi/ml [^{35}S] methionine (specific activity, 1000 Ci/mmol; Amersham, Buckinghamshire, UK) for 2 hours at 37°C. Cells were lysed on ice at $5x10^7$/ml, in lysis buffer containing 0.5% Triton X-100, 300 mM NaCl, 50 nM Tris-HCl, pH 7.6, 10 mM iodoacetaminde, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Anti-CD18 mAB or rat IgG (control) was bound to protein A-Sepharose 4B (Sigma, St. Louis, MO) at a ratio of 3 mg of antibody to 1 ml of packed beads, by using a polyvalent goat antiserum against rat Ig. Immunoabsorbents were incubated with cell lysates for 2 hours in the cold and washed with immunoprecipitation buffer, which was identical to the lysis buffer except that 0.15% Triton was used instead of 0.5%. After

thorough washing the bound material was eluted from the beads with 50 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol. The sample was analyzed by reducing SDS-PAGE (7% polyacrylamide; exposure time, 8 days). As seen in Fig. 2, the anti-CD18 mAb, but not the control reagent (rat IgG), precipitated the α- and β-chains of the LB cell LFA-1 integrin molecule in LB cells.

EXAMPLE 3

BALB/c mice were injected subcutaneously (s.c.) in the left flank with $3x10^6$ LB T cell lymphoma cells. Two hours after the s.c. inoculation of LB cells, and then every other day until the end of the experiment, some of the mice were injected intravenously (i.v.) with the 50% ammonium sulfate fraction (0.5 mg of protein) of anti-CD18 mAb (M18/2) or anti-CD11a mAb (M7/14) or with the same amount of one of the following control mAbs: anti-CD11b (M1/70), anti-CD3 (145-2C11) or anti-D^d (35-5-8C). On day 12 the mice were killed and their spleens and axillary and brachial lymph nodes were removed. Half of the spleens and lymph nodes derived from each treatment group were examined histologically (see Fig. 3), whereas cell suspensions from the other half were simultaneously tested using the proliferation assay (see Fig. 4).

Fig. 3 shows the histological examination of spleen and lymph nodes of the treated mice. Portions of the spleens and axillary and brachial lymph nodes were fixed in 10% buffered formalin and imbedded in paraffin and 5-μm section were stained with hematoxylin and eosine. As seen, in spleens of mice injected with LB cells (Fig. 3b), the normal structure of the spleen was completely effaced: the compartmentalized red and white pulps of the normal spleen were replaced by the infiltrating nuclear vesiculated LB cells, with many of them presenting mitotic figures. In contrast, in mice inoculated with LB cells and injected i.v. with anti-CD18 mAb (Fig. 3c),

the normal organization of the spleen was preserved and its structure completely resembled the spleen of normal mice, indicating that anti-CD18 mAb blocks the infiltration of LB cells into the spleen. In mice injected with LB cells and then with either an anti-CD11a mAb or a control mAb (derived from the same source (rat) and expressing the same isotope (IgG2a)), the structure of the spleen was identical to that of the spleens of the mice inoculated with LB cells alone, i.e. these mAbs failed to protect the spleens from invasion of LB cells.

The lymph nodes' architecture of mice inoculated with LB cells was also completely effaced showing enlargement and heavy infiltration by the lymphoma cells (Fig. 3e). However, in this case, i.v. injection of anti-CD18 mAb did not block the invasion of nuclear vesiculated LB cells into the lymph nodes which were totally effaced by the invading cells (Fig. 4f). It is to be noted that local s.c. injection of anti-CD18 mAb near the axillary or brachial lymph nodes of the mice injected with LB cells at a different and remote site did however, partially block the proliferation of infiltrating LB cells into these organs (not shown).

The histology analysis of the spleens of mice inoculated with LB cells was confirmed by a corresponding [^{3}H]-Thymidine incorporation assay.

Spleens and axillary and brachial lymph nodes were removed from the LB cell-inoculated mice and cell suspensions were prepared in RPMI 1640. The cells were incubated (5% CO_2 atmosphere, 37°C) for 15 hours in U-shaped 96-well microplates (Nunc, Roskilde, Denmark) at $2x10^5$ cells/well). The cells were pulsed with 1 μCi of [^{3}H]Thymidine (specific activity, 5 Ci/mmol; Nuclear Research Center, Negev, Israel) and were harvested with a Titertek cell harvester (Flow Laboratories, Irvine, Scotland, UK) 20 hours later. The incorporation of [^{3}H]Thymidine was determined with a beta counter (Betamatic; Kontron, Montigny le Bertonneux, France). Results are expressed as cpm (mean ± SD). Each experiment is representa-

tive of at least two experiments, showing similar results. Control experiments demonstrated that in these mice, the only cells proliferating in the spleen and lymph nodes are the inoculated LB cells and not local cells (results not shown). As seen in Fig. 4, a strong cell proliferation (indicated by ^{3}H-Thymidine uptake) was revealed in spleen and lymph node cell suspensions of the mice injected with LB cells, whereas the cell proliferation of the corresponding lymph node organs derived from normal mice was very low. i.v. injection of anti-CD18 mAb blocked the proliferation of LB cells invading the spleen, but not those invading the lymph nodes. On the other hand, the proliferation in the spleens of the mice injected with LB cells and also with an anti-CD11a mAb or a different control mAb (anti-CD11b, anti-CD3, anti-D^d and anti-ICAM-1) was not blocked. The injection of anti-CD18 mAb purified with protein G or its $F(ab')_2$ fragments also blocked the proliferation of s.c. inoculated LB cells in the spleens of these mice, indicating that the elimination of LB cells by the anti-CD18 mAb is not by C-dependent lysis or antibody-dependent cellular clearance.

EXAMPLE 4

LB cells were removed from the ascitic fluid of 6 to 8 week old female BALB/c mice. Some of these cells (10^6/ml) were incubated (5% CO_2 atmosphere, 37°C) with PMA (20 ng/ml) for 16 hours. Ammonium sulfate precipitated anti-CD18 mAb (M18/2, rat IgG2a) was added (140 μg) to $1x10^6$ prewashed PMA activated or nonactivated LB cells. The bound antibody was detected with anti-rat IgG antibody [$F(ab')_2$, Sigma, St. Louis, MO] conjugated to fluorescein isothiocyanate (FITC), following analysis by FACS 440 (Bacton-Dickenson, Mountain View, CA) tuned to 488 nm.

As seen in Fig. 5A, LB cells express the CD44 (PgP-1) adhesion molecule on their cell surface. Double staining of the LB cells with anti-CD18 and anti-CD44 antibodies was performed by adding a mixture of

FITC-conjugated rat anti-mouse CD18 mAb (C71/16, IgG2a, PherMingen, San Diego, CA) and phycoerythrin-conjugated rat anti-mouse CD44 mAb (IM7, IgG2b, FarMingen) to the cell suspension. The cells were kept 30 mins. on ice, washed and analyzed by FACS, as indicated above. As seen in Fig. 5B the vast majority of the LB cells simultaneously expressed both the CD18 and the CD44 adhesion molecules on their cell surface.

EXAMPLE 5

Immunoprecipitation of ^{35}S-methionine labeled LB extract with anti-CD44 mAb and subsequent gel electrophoresis was performed as described in Example 2 above. The results, seen in Fig. 6, confirmed the presence of CD44 adhesion molecules on LB cells and revealed that these cells expressed the 85-95 kDa isoform (termed standard CD44-sCD44) of this adhesion molecule).

EXAMPLE 6

Female BALB/c were inoculated s.c. with $3x10^6$ LB cells and two hours later some of the mice were injected s.c. near the draining lymph nodes with a 50% ammonium sulfate fraction of an anti-CD44 mAb. The antibody injection was repeated every other day until termination of the experiment on day 12. The spleens and lymph nodes of the treated mice were removed and subjected to histological examination and cell suspensions of these organs were prepared and used for proliferation assays, as described in Example 3 above. Other mice were also inoculated s.c. with LB cells and then received i.v. injections of anti-CD18 mAb as described in Example 3 above. As seen in Fig. 7, the structure of the lymph nodes of mice receiving s.c. injections of anti-CD44 mAb (D in Fig.) was completely normal as compared to their spleens (H in Fig.) which were heavily invaded by LB cells and were identical to the spleens of mice inoculated with LB

cells alone (F in Fig.). In agreement with the results of the experiments described in Example 3 above, i.v. injection of anti-CD18 mAb in the mice inoculated with LB cells preserved the normal structure of their spleen (G in Fig.) but not of their lymph nodes (C in Fig.).

As in Example 3 above, the histological findings were confirmed by [^{3}H]-thymidine incorporation assays which, in this case, as seen in Fig. 8, demonstrated that anti-CD44, but not the control mAb anti-CD4 blocked the proliferation of lymph node-infiltrating LB cells but not that of spleen-infiltrating LB cells. It is to be noted, that when anti-CD44 was injected i.v. rather than s.c., the proliferation of LB cells invading the lymph node was only partially inhibited.

EXAMPLE 7

The ability of LB cells to form *in vitro* aggregates with cells derived from normal spleens or lymph nodes was examined by incubation of the LB cells with spleen or lymph node cells in a 24-well microplate (Cel-Cult; Sterilin, Feltham, UK), at various LB cell to target cell ratios, for 16 hours in a CO_2 incubator. Aggregate formation was examined under an inverted microscope. Total cell concentration was determined in a sample from each well after the aggregates were broken by repeated forceful pipetting. The remaining cell suspension was then filtered through Polymon 120-T mesh (60 μm; Swiss Silk, Zurich, Switzerland). Cell aggregates were trapped on the mesh; the non-aggregated cells that passed through the mesh were collected and their concentration was determined again. Cell count was performed either with a light microscope or with a Coulter counter (Coulter Counter Electronics Ltd., Luton, UK). The percentage of cell aggregation was calculated by using the following formula: 100 - cell concentration (cells/ml) after filtration/cell concentration before filtration.

As seen in Fig. 9, the LB cells formed aggregates with spleen cells (A in the figure) at various spleen cell to LB cell ratios but did not form aggregates with lymph node cells (B in the figure) at any lymph node cell to LB cell ratio. Addition of the anti-CD18 mAb to the spleen cell-LB cell suspension blocked the aggregate formation between LB cells and spleen cells by 82% (not shown). Furthermore, anti-CD18 mAb injected i.v. blocked *ex vivo* aggregates formed spontaneously in the spleen after s.c. inoculation of LB cells whereas the other control antibodies did not.

EXAMPLE 8

BALB/c mice were inoculated with RAW 117-H-10 lymphosarcoma cells s.c. and then divided into four groups:

(i) mice receiving no further treatment (control);

(ii) mice receiving a subsequent i.v. injections with anti-VACM-1 mAb (MW2.7);

(iii) mice receiving a subsequent i.v. injections with anti-VLA-4 (PS/1) mAb; and

(iv) mice receiving subsequent i.v. injections with a combination of anti-VACM-1 and anti-VLA-4 mAbs.

Mice from the various groups were killed on day 14, their livers were removed and analyzed histologically. As seen in Fig. 10, the H-10 cells infiltrated the liver parenchima via the blood vessels (the dark area in the pictures) of livers from the control mice (Fig. 10A) in the mice injected with anti-VACM-1 mAb (Fig. 10B) and in the livers of mice injected with the anti-VLA-4 mAb (Fig. 10C). In contrast, livers of H-10 cell innoculated mice which were i.v. injected with a combination of the anti-VACM-1 and anti-VLA-4 mAbs was almost totally protected from invasion by the lymphoma (Fig. 10D).

Proliferation of the lymphosarcoma H-10 cells in the liver was also measured by ^{3}H-thymidine incorporation (as described in Example 3 above). BALB/c mice were inoculated s.c. with either cells of the parent line RAW 117-P or with cells of the lymphosarcoma RAW 117-H-10. The mice inoculated with the H-10 cells were divided into the same groups as described above and received either no further treatment, i.v. injections with anti-VACM-1 mAb or anti-VLA-4 mAb or a combination of both antibodies. As seen in Fig. 11, while each of the above injected antibodies alone could not block the liver invasion by H-10 cells, the proliferation of the lymphosarcoma cells in livers of mice receiving an injection of the two antibodies was almost totally blocked.

These results indicate that both the lymphosarcoma VLA-4 adhesion molecules and the complementary liver associated VACM-1 molecules must be blocked in order to protect the liver from infiltration by the lymphosarcoma H-10 cells.

EXAMPLE 9

LB cells were labeled with [^{3}H]-Thymidine and their *in vitro* binding ability to plastic coated hyaluronic acid (HA) was tested as follows: A flat bottom 96-well microplate (Nunc, Roskilde, Denmark) was coated with 0.5 mg/well HA (Sigma) or other glycosaminoglycans. After 16 hours incubation at room temperature, the microplate was washed three times with phosphate buffered saline. LB cells (10^6/ml) were incubated (5% CO_2, 37°C) with 5 μCi/ml [^{3}H]Thymidine (specific activity 5 Ci/mmole; Nuclear Research Center, Negev, Israel) in the presence of 20 ng/ml PMA. After 16 hours, radiolabeled LB cells were washed and resuspended in RPMI 1640 containing 10% fetal calf serum and 3x10^4 labeled cells were added to each HA coated well. In some of the experiments the HA coated microplate also contained tested mAbs (300 μg protein/well), the enzyme hyaluronidase (100

μg/well) or other related enzymes at concentrations of equivalent specific activity. The bound cells were washed after one hour of incubation (5% CO_2, 37°C) and harvested with a Titertek cell harvester (Flor Laboratories, Irvine, Scotland). Radiolabeled bound cells were counted with a beta counter (Betamatic, Kontron, Montigny le Bertonneux, France). Percent adhesion was calculated according to the following equation: bound cpm per well divided by total cpm added to each well x 100. Results of the binding assay showed that whereas non-activated LB cells did not bind to HA (results not shown), LB cells activated by PMA (after incubation with 20 ng per/ml PMA) demonstrated extensive binding to HA (with a maximum level of about 40% binding at a concentration of 0.5 ml HA per well). Equivalent amounts of other glycosaminoglycans (heparin, heparan sulfate, and chondroitin sulfate) did not bind the activated cells (Fig. 12-1). The binding of the activated LB cells to HA was blocked by the anti-CD44 monoclonal antibody and its $F(ab)_2$ and Fab fragments (Fig. 12, 2,3) but not by the anti-CD18 or anti-CD4 mAbs (Fig. 12-2). The binding of the activated LB cells to HA was also blocked by the enzyme hyaluronidase (at a concentration of 100 μg/well) whereas the enzymes heparinase and chondroitinase AC, at equivalent specific activities did not block the binding of LB cells to HA (Fig. 12-4).

EXAMPLE 10

BALB/c mice were inoculated s.c. with $3x10^6$ LB cells. Some of the mice were also locally s.c. injected with hyaluronidase (0.5 mg/mouse) near the lymph node or with other enzymes at doses adjusted to an equivalent specific activity. Enzyme injection was repeated every other day until termination of the experiment. The mice were killed on day 12, their lymph nodes and spleens were removed, cell suspensions were prepared and the level of their proliferation was measured by ^3H-thymidine incorporation

assay, as described in Example 3 above. As seen in Fig. 13, the invasion by proliferating LB cells was inhibited in lymph nodes of mice receiving hyaluronidase injection whereas the lymph nodes of mice receiving injection of other related enzymes, at an equivalent specific activity, were invaded by proliferating LB cells. In contrast, the spleens of mice receiving a local s.c. injection of hyaluronidase were invaded by proliferating LB cells.

These results were confirmed by histological analysis of the spleen and lymph nodes of the mice from the various above indicated groups. As seen in Fig. 14, the normal architecture of the spleen and lymph node of BALB/c mice inoculated s.c. with LB cells were almost completely effaced by the invading lymphoma cells (Fig. 14A). However, in mice inoculated with LB cells and also subcutaneously injected with hyaluronidase near the lymph node, the examined lymph node did not contain invading lymphoma cells whereas the spleen of these mice was invaded by such cells (Fig. 14B).

CLAIMS:

1. An anti-invasion agent for blocking invasion of tumor cells into the spleen, comprising an effective amount of an active agent being one or more of the group consisting of:

(a) anti-CD18 antibody;

(b) a fragment of the antibody of (a) comprising an antigen binding site thereof; and

(c) a modified molecule derived from (a) and (b) comprising an antigen binding site having the same binding specificity as said antibody or fragment.

2. An anti-invasion agent, for blocking invasion of tumor cells into lymph nodes, comprising an effective amount of an active agent being one or more of the group consisting of:

(a) anti-sCD44 antibody;

(b) a fragment of the antibody of (a) comprising an antigen binding site thereof; and

(c) a modified molecule derived from (a) and (b) comprising an antigen binding site having the same binding specificity as said antibody or fragment.

3. An anti-invasion agent for blocking invasion of tumor cells into lymph nodes, comprising an effective amount of an active agent being one or more of the group consisting of:

(a) hyaluronidase;

(b) hyaluronidase fragment capable of breaking down hyaluronic acid;

(c) an agent other than hyaluronidase or said fragment capable of breaking down hyaluronic acid;

(d) hyaluronic acid; and

(e) analogs of hyaluronic acid capable of binding to the sCD44 antigen.

4. An anti-invasion agent for blocking invasion of tumor cells into the liver, comprising a synergistic combination of a first and a second agent; said first agent being one or more of the group consisting of:

(a) anti-VLA-4 antibody;

(b) a fragment of the antibody of (a) comprising an antigen binding site thereof; and

(c) a modified molecule derived from (a) and (b) comprising an antigen binding site having the same binding specificity as said antibody or fragment;

said second agent being one or more of the group consisting of:

(d) an anti-VACM-1 antibody;

(e) a fragment of the antibody of (a) comprising an antigen binding site thereof; and

(f) a modified molecule derived from (a) and (b) comprising an antigen binding site having the same binding specificity as said antibody or fragment.

5. A method of cancer treatment, comprising administering to the patient in need an effective amount of an anti-invasion agent being one or more of the group consisting of:

(a) anti-CD18 antibody;

(b) a fragment of the antibody of (a) comprising an antigen binding site thereof; and

(c) a modified molecule derived from (a) and (b) comprising an antigen binding site having the same binding specificity as said antibody or fragment.

6. A method of cancer treatment, comprising administering to the patient in need an effective amount of an anti-invasion agent being one or more of the group consisting of:

(a) anti-sCD44 antibody;

(b) a fragment of the antibody of (a) comprising an antigen binding site thereof; and

(c) a modified molecule derived from (a) and (b) comprising an antigen binding site having the same binding specificity as said antibody or fragment.

7. A method of cancer treatment, comprising administering to the patient in need an effective amount of an anti-invasion agent being one or more of the group consisting of:

(a) hyaluronidase;

(b) hyaluronidase fragment capable of breaking down hyaluronic acid;

(c) an agent other than hyaluronidase or said fragment capable of breaking down hyaluronic acid;

(d) hyaluronic acid; and

(e) analogs of hyaluronic acid capable of binding to the sCD44 antigen.

8. A method of cancer treatment, comprising administering to the patient in need an effective amount of an anti-invasion agent being one or more of the group consisting of:

(a) anti-VLA-4 antibody;

(b) a fragment of the antibody of (a) comprising an antigen binding site thereof; and

(c) a modified molecule derived from (a) and (b) comprising an antigen binding site having the same binding specificity as said antibody or fragment;

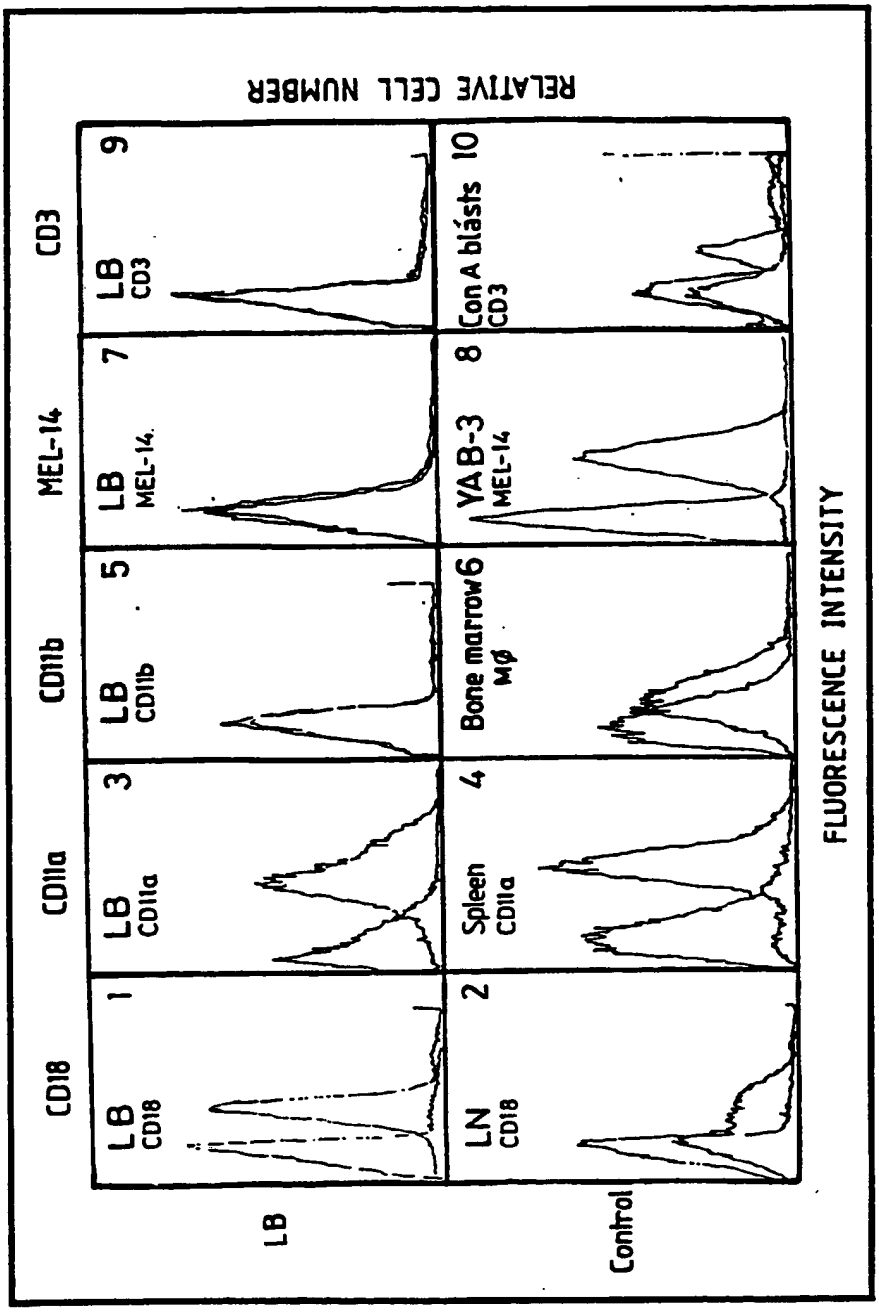
said second agent being one or more of the group consisting of:

(d) an anti-VACM-1 antibody;

(e) a fragment of the antibody of (a) comprising an antigen binding site thereof; and

(f) a modified molecule derived from (a) and (b) comprising an antigen binding site having the

same binding specificity as said antibody or fragment.


RELATIVE CELL NUMBER
FLUORESCENCE INTENSITY
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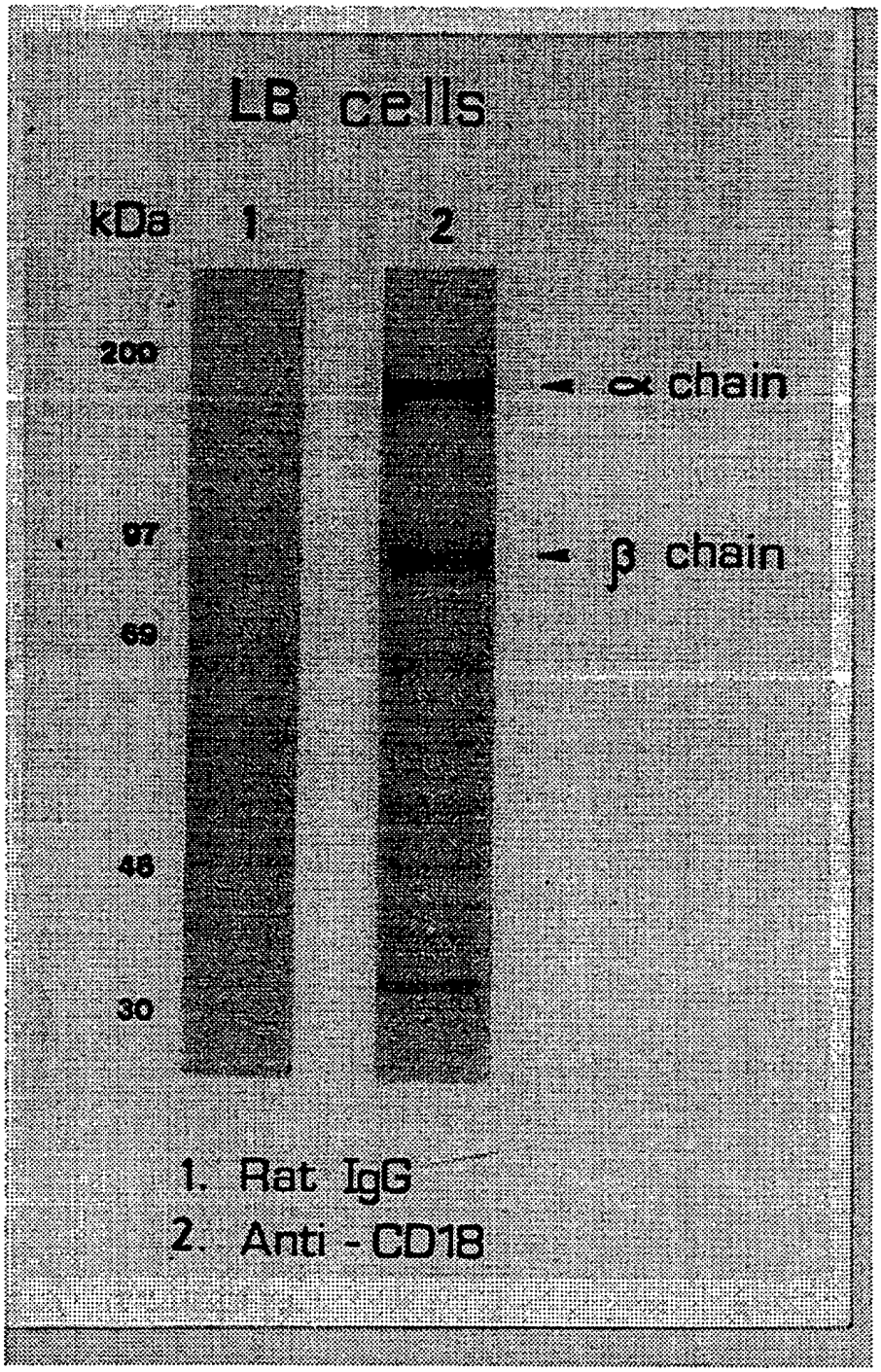
CD11a
CD11b
MEL-14
CD3
LB
Control
1
LB CD18
2
LN CD18
3
LB CD11a
4
Spleen CD11a
5
LB CD11b
6
Bone marrow Mφ
7
LB MEL-14
8
YAB-3 MEL-14
9
LB CD3
10
Con A blasts CD3

FIG. 1


LB cells
kDa
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2
200
97
69
46
30
α chain
β chain
1. Rat IgG
2. Anti - CD18

FIG.2

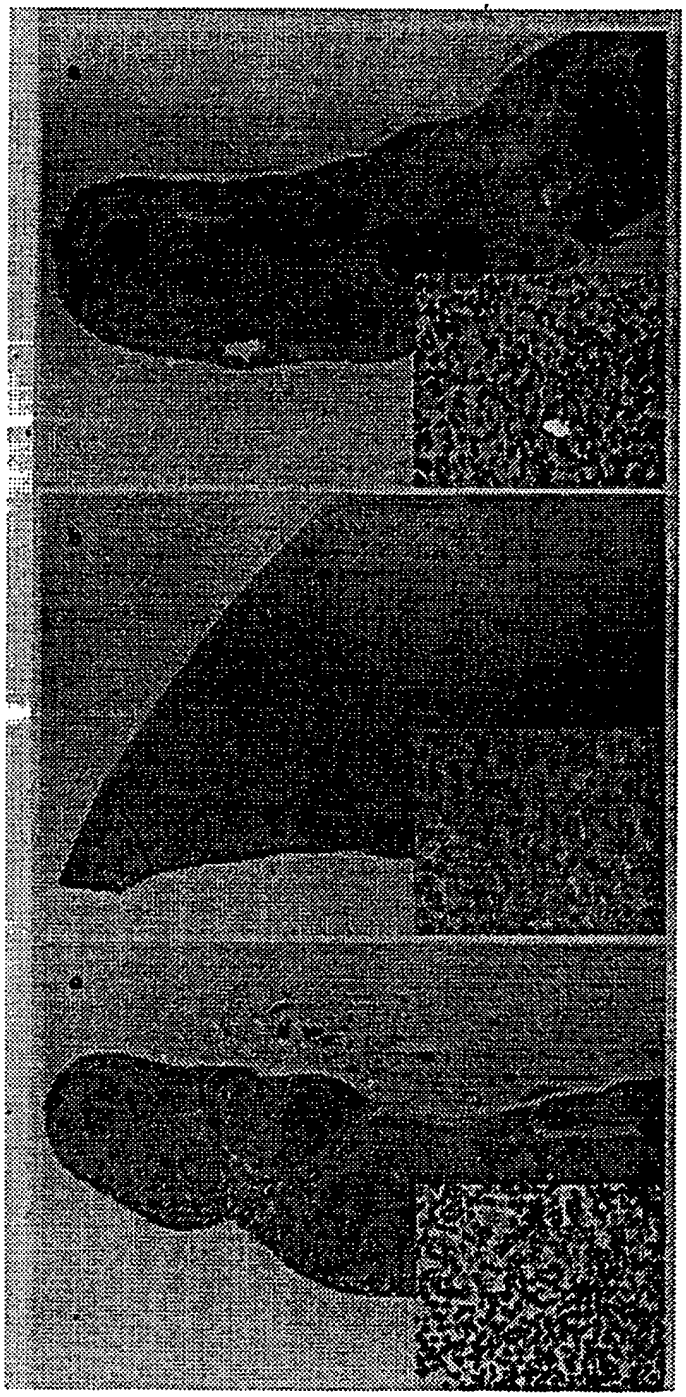
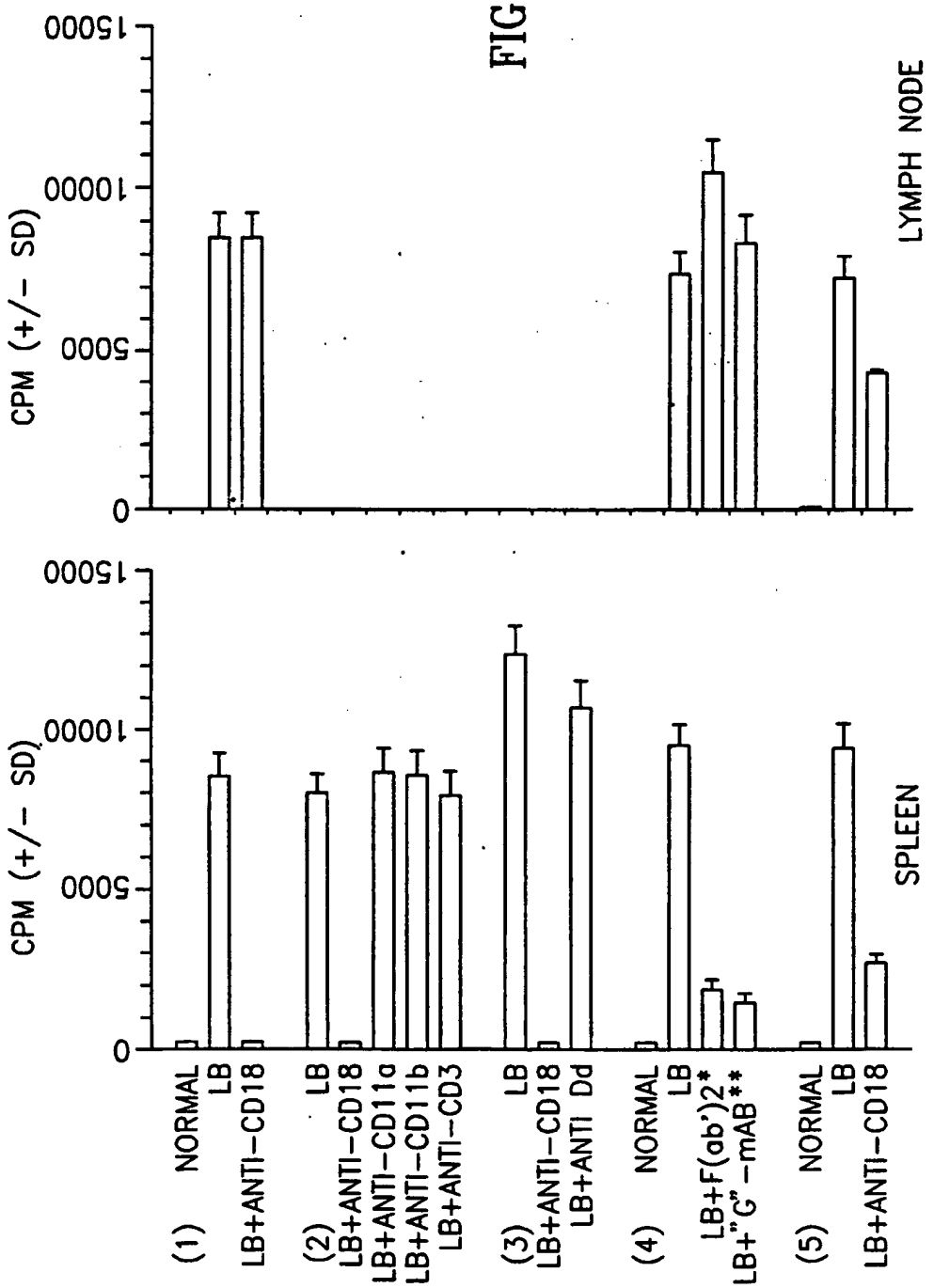


FIG.3A

FIG.3B

FIG.4


15000
10000
5000
0
CPM (+/- SD)
LYMPH NODE
SPLEEN
(1) NORMAL
LB
LB+ANTI-CD18
(2)
LB
LB+ANTI-CD18
LB+ANTI-CD11a
LB+ANTI-CD11b
LB+ANTI-CD3
(3)
LB
LB+ANTI-CD18
LB+ANTI Dd
(4) NORMAL
LB
LB+F(ab')2*
LB+"G"-mAB**
(5) NORMAL
LB
LB+ANTI-CD18

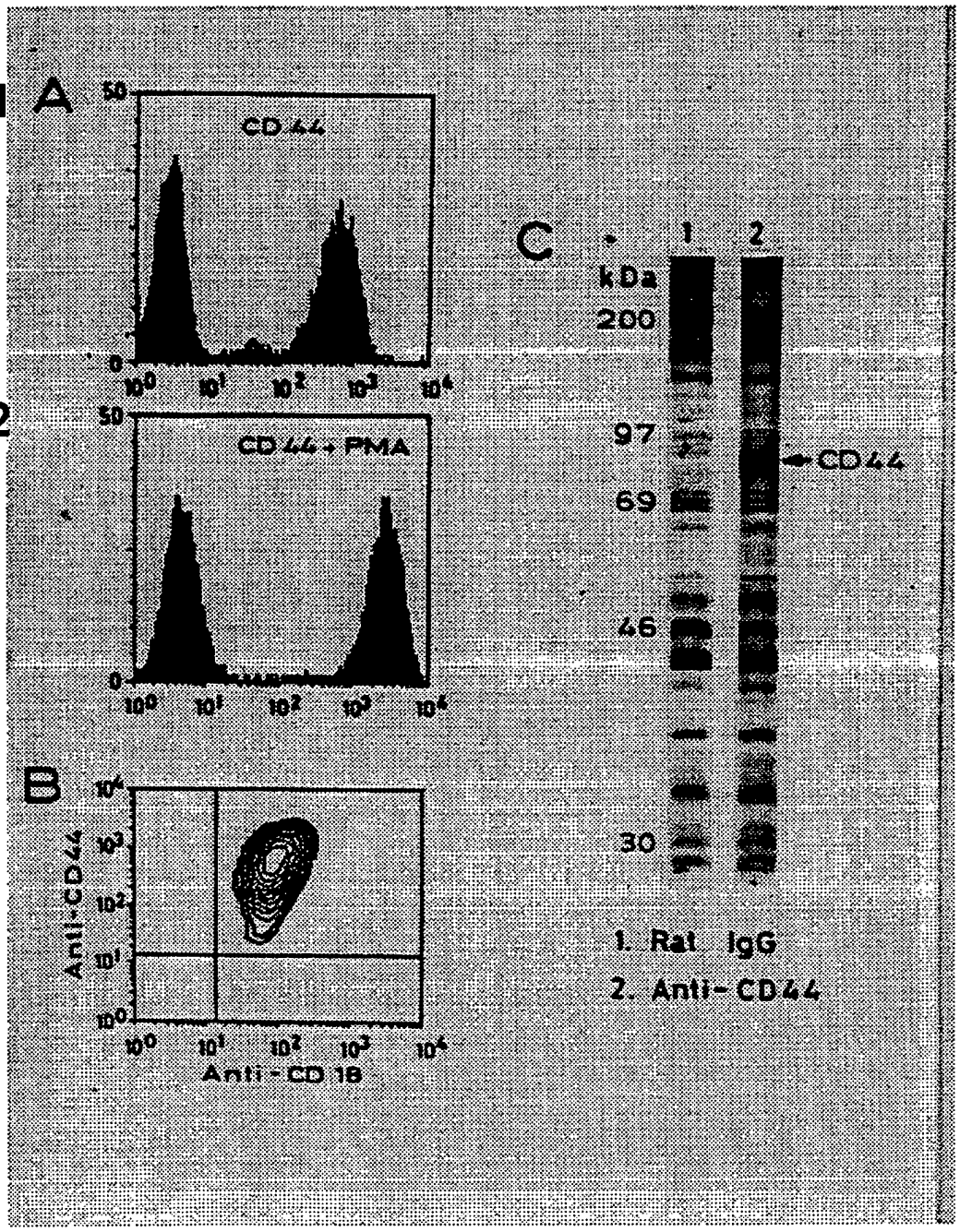

A1
A
CD 44
A2
CD 44 + PMA
B
Anti-CD 44
Anti-CD 18
C
kDa
200
97
69
46
30
1
2
CD44
1. Rat IgG
2. Anti-CD44

FIG.5 FIG.6

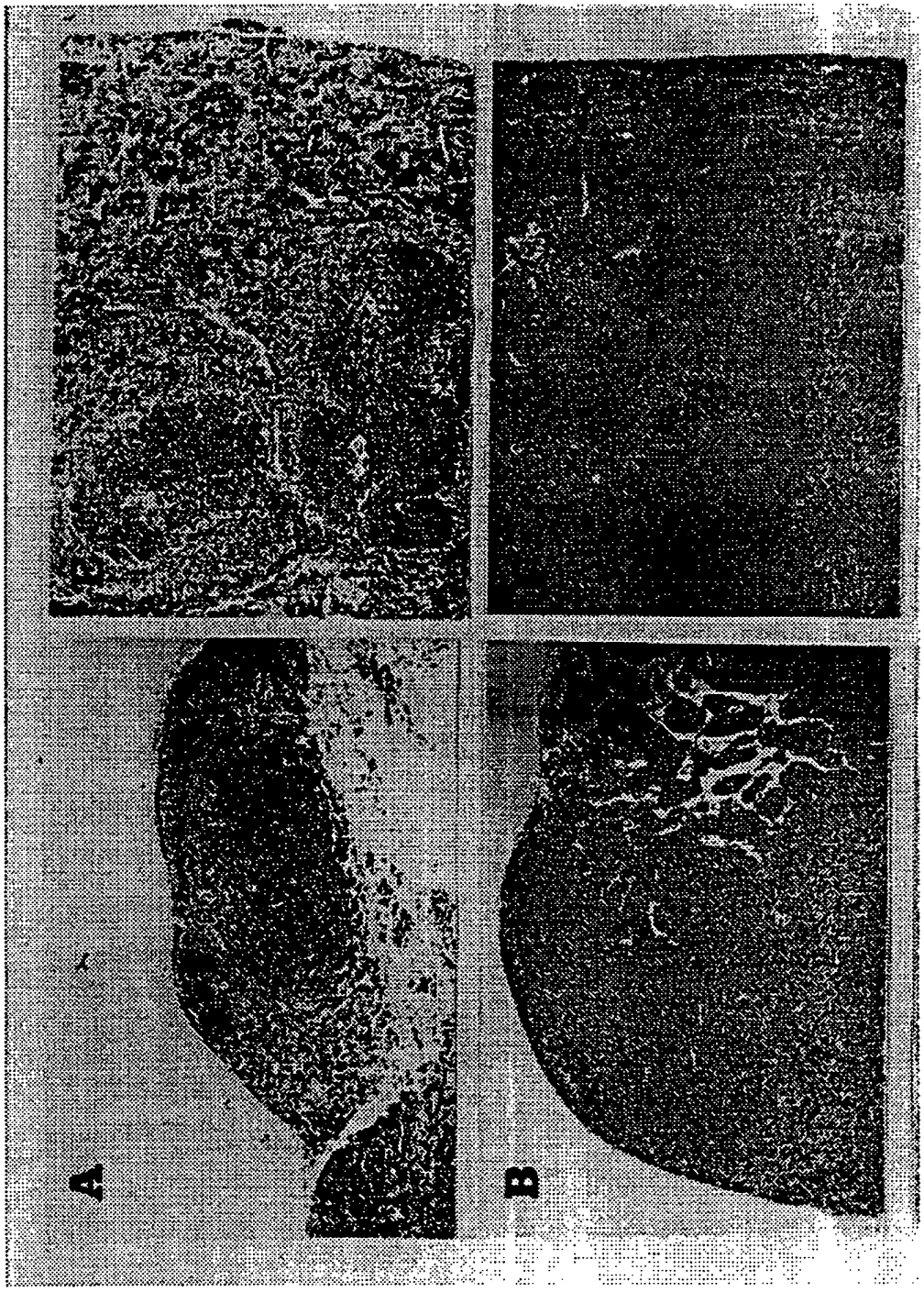


FIG.7A

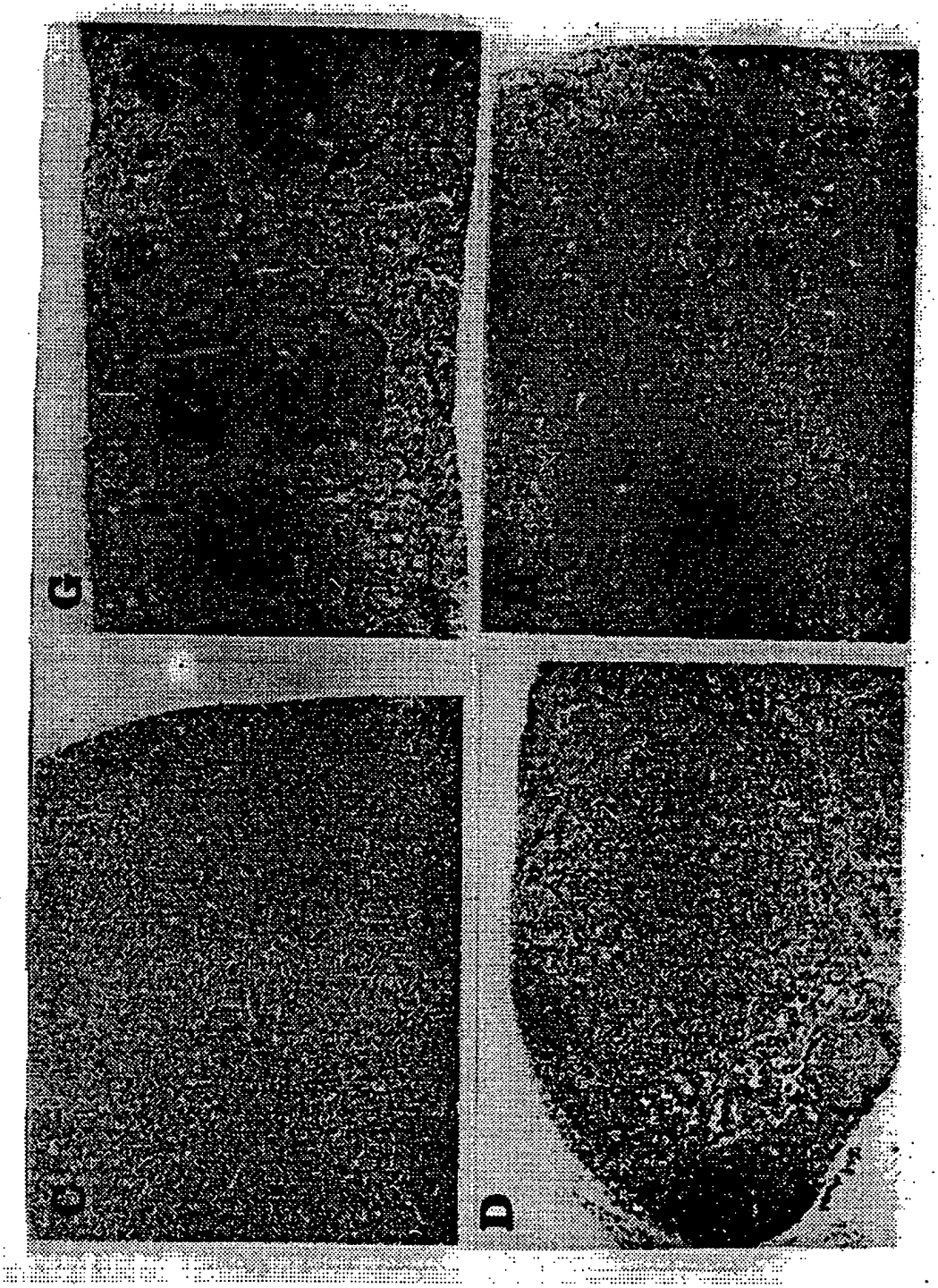
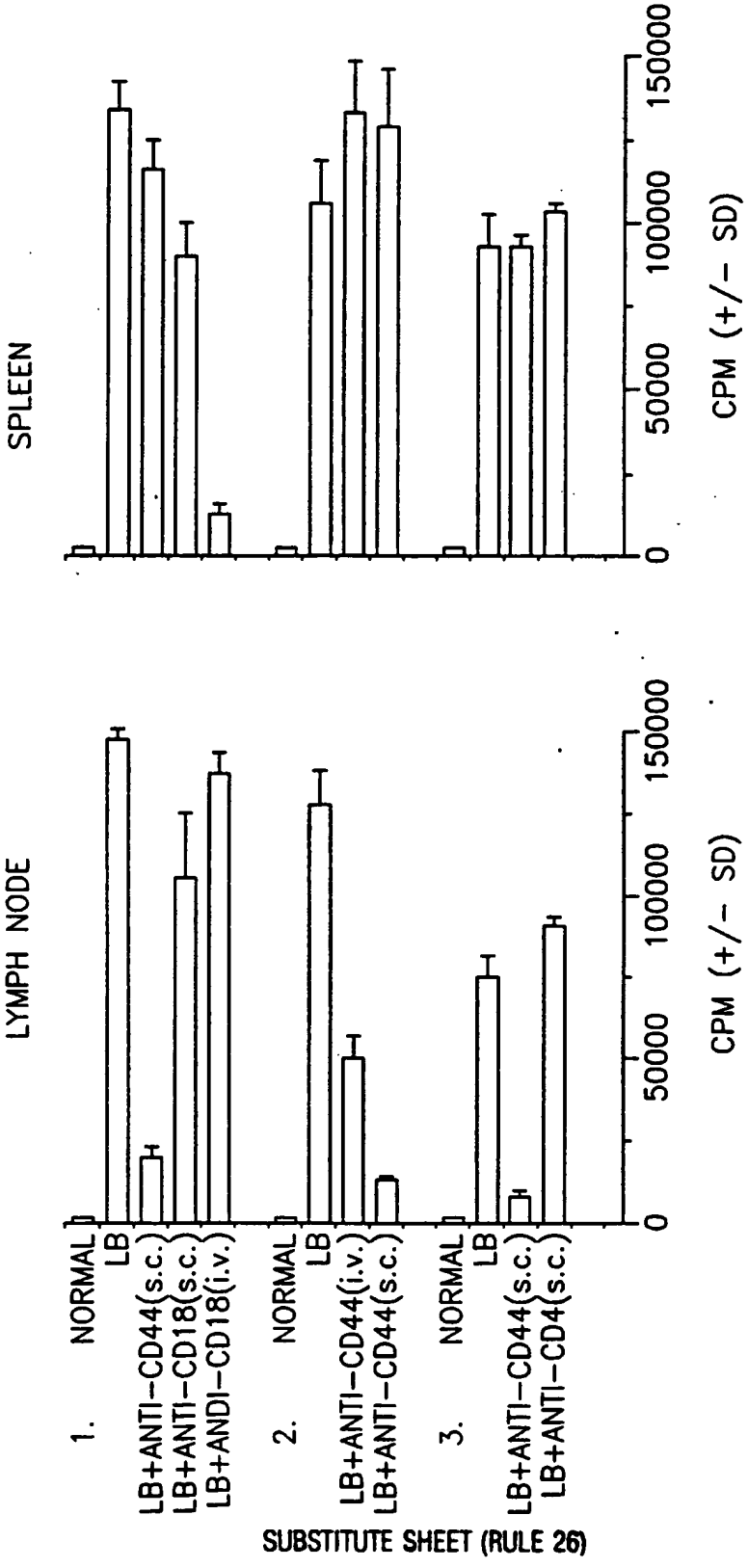


FIG. 7B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CPM (+/- SD)
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50000
100000
150000
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LB
LB+ANTI-CD44(s.c.)
LB+ANTI-CD18(s.c.)
LB+ANDI-CD18(i.v.)
2. NORMAL
LB
LB+ANTI-CD44(i.v.)
LB+ANTI-CD44(s.c.)
3. NORMAL
LB
LB+ANTI-CD44(s.c.)
LB+ANTI-CD4(s.c.)

FIG.8

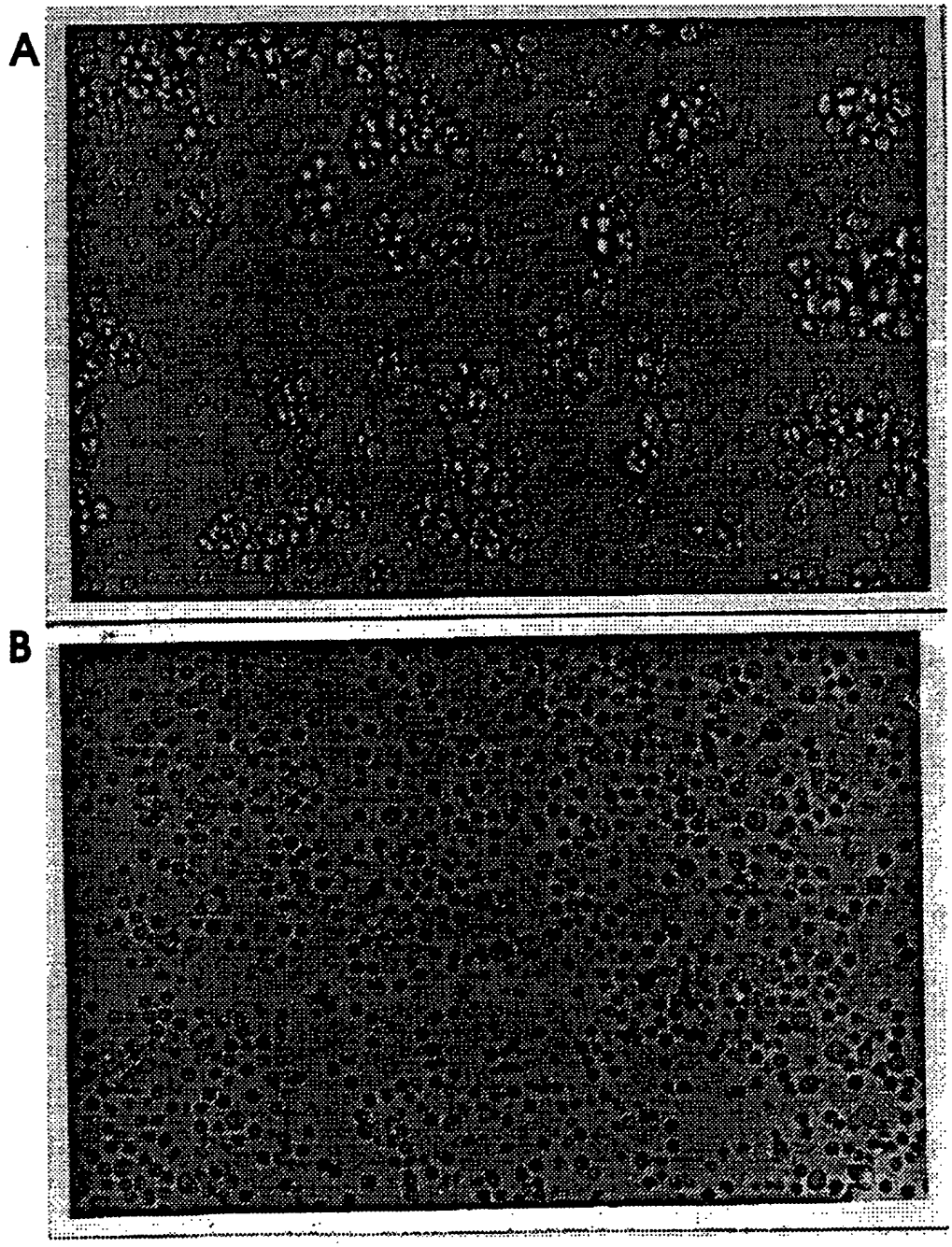


FIG.9

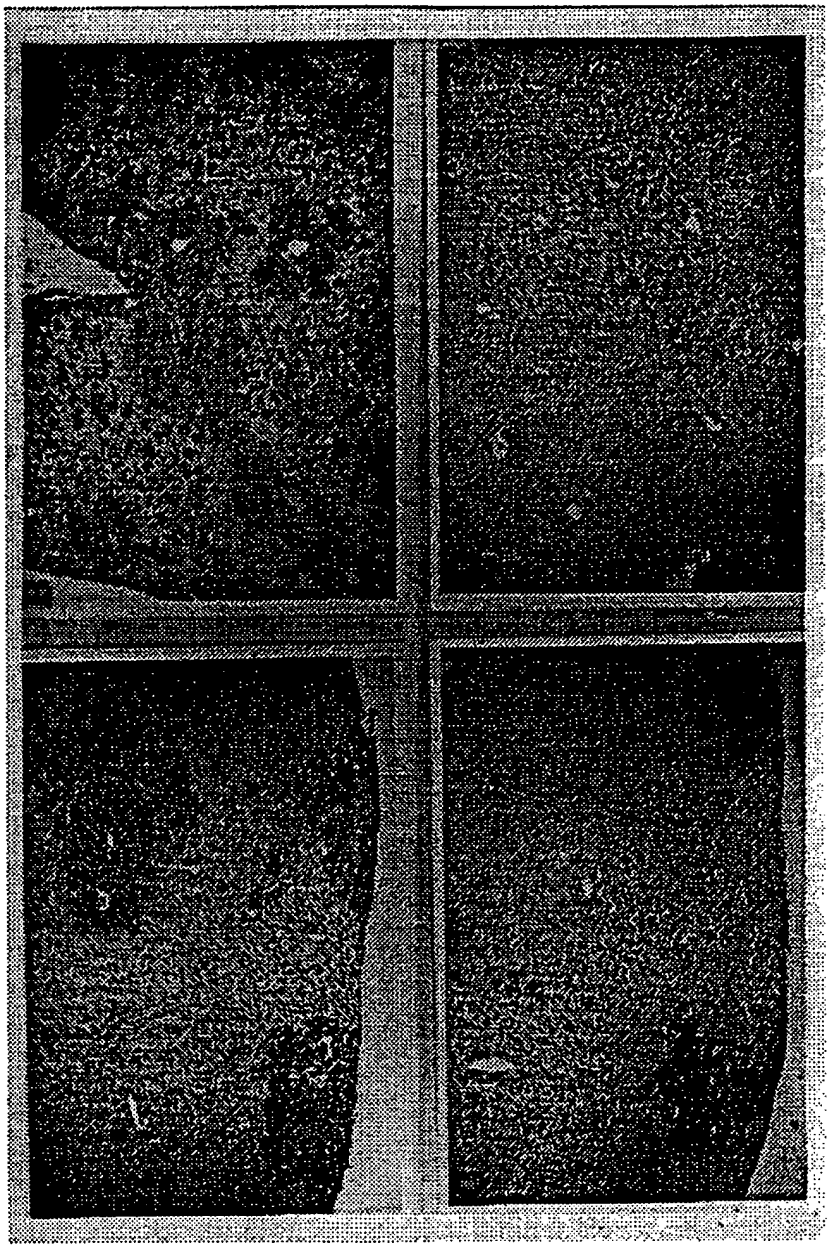


FIG.10

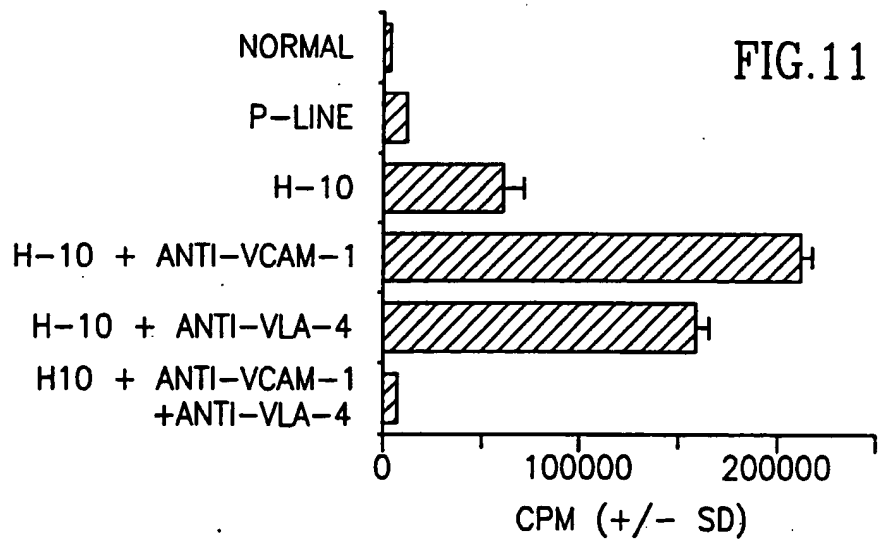

NORMAL
P-LINE
H-10
H-10 + ANTI-VCAM-1
H-10 + ANTI-VLA-4
H10 + ANTI-VCAM-1
+ANTI-VLA-4
0
100000
200000
CPM (+/- SD)

FIG.11

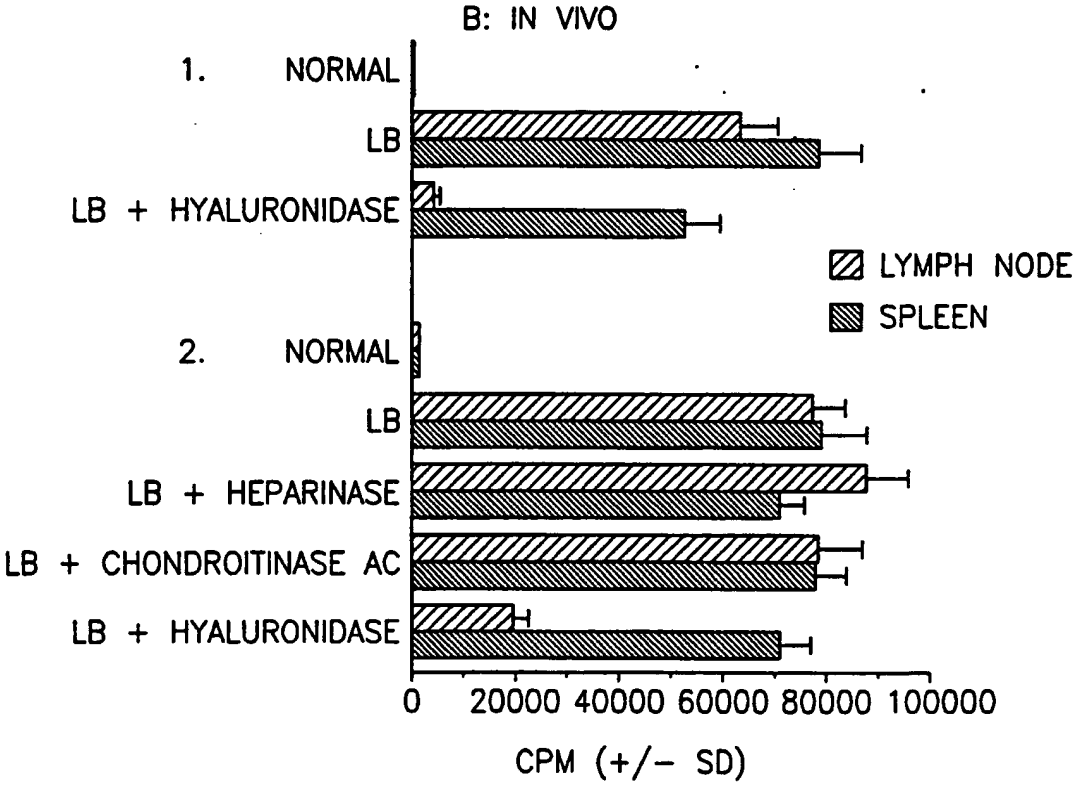
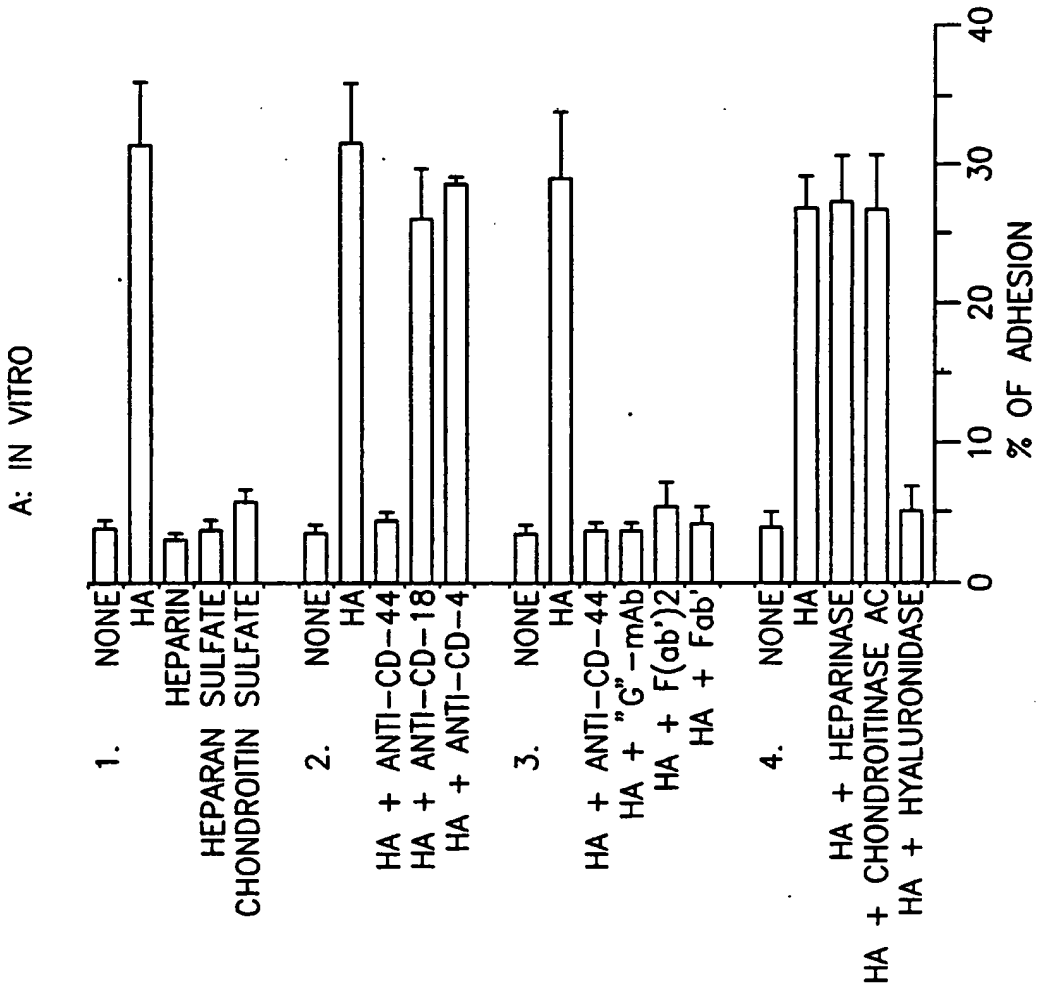

B: IN VIVO
1. NORMAL
LB
LB + HYALURONIDASE
LYMPH NODE
SPLEEN
2. NORMAL
LB
LB + HEPARINASE
LB + CHONDROITINASE AC
LB + HYALURONIDASE
0
20000
40000
60000
80000
100000
CPM (+/- SD)

FIG.13

FIG.12


A: IN VITRO
% OF ADHESION
0
10
20
30
40
1.
NONE
HA
HEPARIN
HEPARAN SULFATE
CHONDROITIN SULFATE
2.
NONE
HA
HA + ANTI-CD-44
HA + ANTI-CD-18
HA + ANTI-CD-4
3.
NONE
HA
HA + ANTI-CD-44
HA + "G"-mAb
HA + F(ab')2
HA + Fab'
4.
NONE
HA
HA + HEPARINASE
HA + CHONDROITINASE AC
HA + HYALURONIDASE

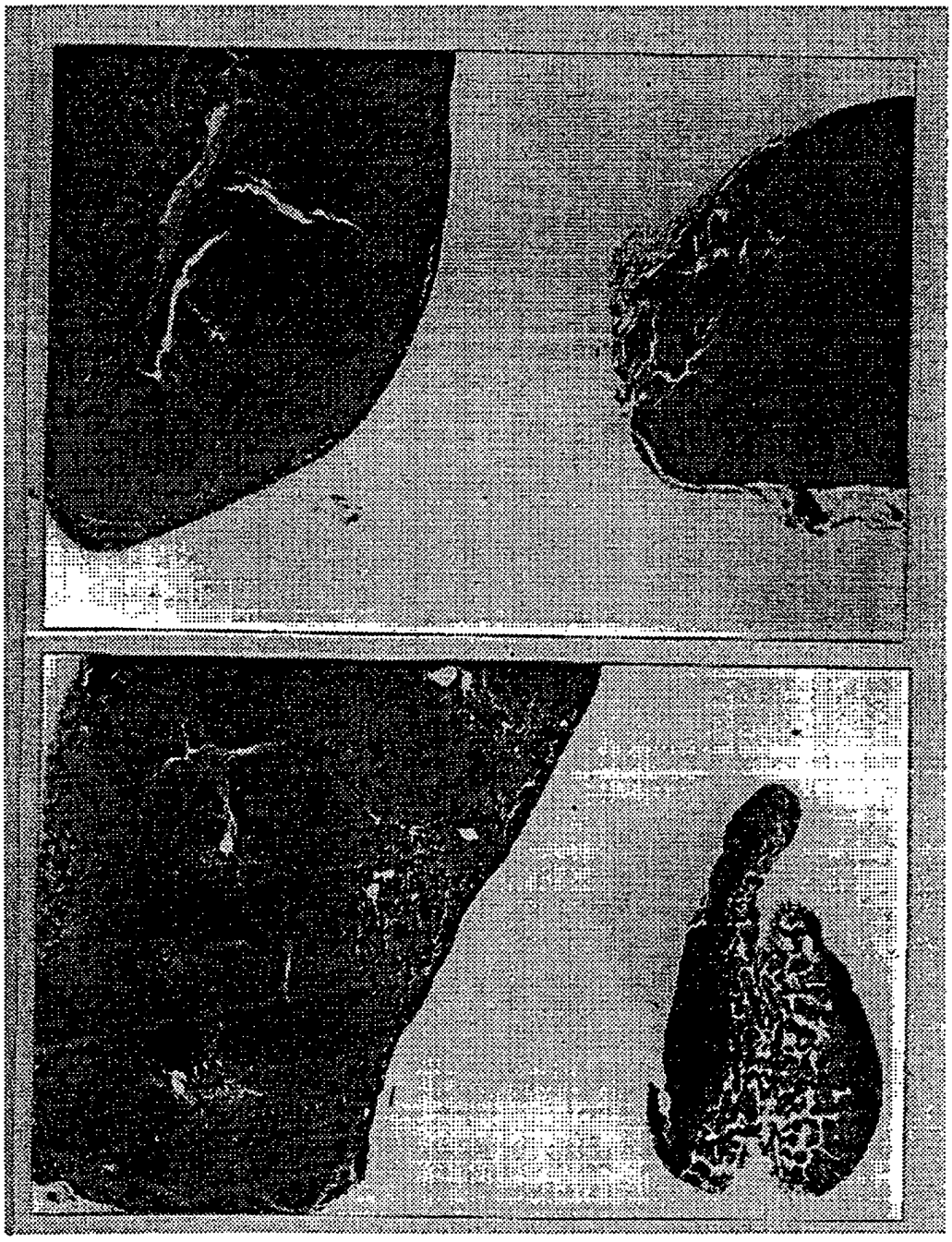


FIG.14

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(74) Agent: NEIMARK, Sheridan; Browdy and Neimark, Suite 300, 419 7th Street, N.W., Washington, DC 20004 (US).

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(57) Abstract

Tumor metastasis is inhibited by the use of active agents which block infiltration of tumor cells into secondary target organs. These agents include a number of antibodies and various fragments and derivatives thereof as well as hyaluronidase, hyaluronic acid and analogs thereof.

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GA	Gabon				

INTERNATIONAL SEARCH REPORT

Internati Application No
PCT/US 95/05819

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/395 A61K38/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	THE JOURNAL OF IMMUNOLOGY, vol.150, no.10, 15 May 1993, BALTIMORE, MD, USA pages 4466 - 4477 M. ZAHALKA ET AL. 'Blocking lymphoma invasiveness with a monoclonal antibody directed against the beta-chain of the leukocyte adhesion molecule (CD18).' see abstract --- -/--	1,5

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Date of the actual completion of the international search	Date of mailing of the international search report
11 September 1995	19.12.95
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer NOOIJ, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF CELLULAR BIOCHEMISTRY, SUPPLEMENT, no.16F, , NEW YORK, NY, USA page 177 D. NAOR ET AL. 'Blocking effect of monoclonal antibody directed against the adhesion molecule (CD18) on the invasion of murine LB lymphoma into lymphoid organs.' see abstract X420 ---	1,5
P,X	INTERNATIONAL IMMUNITY, vol.6, no.6, 4, OXFORD, GB pages 917 - 924 M. ZAHALKA ET AL. 'Beta2-integrin dependent aggregate formation between LB T cell lymphoma and spleen cells: Assessment of correlation with spleen invasiveness.' see abstract -----	1,5

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05819

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 5-8
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although these claims are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- claims 1 and 5
- claims 2 and 6
- claims 3 and 7
- claims 4 and 8

See additional sheet PCT/ISA/210

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1 and 5

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US95/05819

FURTHER INFORMATI N C NTINUED FROM PCT/ISA/210

1. Claims: 1 and 5 Blocking of metastasis of the spleen by antibodies (or fragments/derivatives thereof) anti-CD18.

2. Claims: 2 and 6 Blocking of metastasis of the lymph nodes by antibodies (or fragments/derivatives thereof) anti-sCD44.

3. Claims: 3 and 7 Blocking of metastasis of the lymph nodes by hyaluronic acid or hyaluronidase

4. Claims: 4 and 8 Blocking of metastasis of the liver by a combination of antibodies (or fragments/ derivatives thereof) anti-VLA-4 and anti-VCAM-1.